US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for Enumeration of Bacterial Inocula on Carriers (Carrier Counts)

SOP Number: MB-04-04

Date Revised: 04-04-07

Superseded SOP: MB-04-03 - Determining Carrier Counts

EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

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Initiated By: _____ Date: ___/___/___

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Technical Staff

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Approved By: _____ Date: ___/___/___

Print Name:_____
Branch Chief

Effective Date: ___/___/___

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TABLE OF CONTENTS

Contents Page Number

1.0 SCOPE AND APPLICATION:

1.1 The monitoring of the bacterial titer associated with inoculated carriers (e.g., stainless steel, porcelain and glass slides) used in antimicrobial product testing is required by the laboratory. This protocol describes the procedures for the enumeration of test microbes on the carriers used in efficacy testing (see SOPs: MB-05, MB-06, MB-07, MB-09, MB-15).

2.0 DEFINITIONS:

2.1 CFU = Colony Forming Units

2.2 TNTC = Too Numerous to Count

2.3 AOAC =AOAC International

2.4 DI = De-ionized water

2.5 Dilution Blanks = tubes of phosphate buffered dilution water (PBDW)

2.6 10^0 = the tube with the carrier

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.

4.0 CAUTIONS:

4.1 Processing (sonication or vortexing) of selected inoculated carriers will be performed as soon as possible.

4.2 Dilution plating should be performed preferably within 2 hours after the completion of carrier sonication or vortexing. If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5EC until this step can be done.

4.3 For spread plating: ensure that the entire surface of the agar plate is dry before adding inoculum. If necessary, leave the agar plates uncovered in the BSC until the moisture has been completely absorbed into the medium.

5.0 INTERFERENCES:

5.1 Contaminated plates will interfere with the recording of results. Visually inspect

all agar plates prior to use – discard any plates with evidence of contamination. For contamination following the incubation phase, if atypical colonies or contamination are evident that interfere with the enumeration of the test organism, record as a contaminant(s). Data from other dilutions, if the CFUs result in a countable range, may be used to calculate the final CFU/carrier.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Branson Model 2200 Ultrasonic Cleaner (sonicator) or equivalent

7.2 A water bath set at 45-50EC

7.3 Incubator at 36±1EC or another temperature suitable for growth of the target organism

7.4 Refrigerator set at 2-5EC

7.5 20 × 100 mm Petri dishes (total plating area of 58.1 cm^2)

7.6 Plate spinner

7.7 Trypticase Soy Agar (TSA) or Middlebrook 7H9 Agar (M7H9)

7.8 Letheen Broth or Modified Proskauer Beck Medium (MPB)

7.9 Leica Darkfield Quebec Colony Counter

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 For the inoculation of glass slide carriers, the calibration of Eppendorf pipettes is required (refer to SOP QC-19).

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Sonicated or vortexed tubes (see sections 10.1.3, 10.2.1 and 10.3.2) can be stored at 2-5EC until the dilution plating is performed. Plating should be completed

preferably within two hours of the sonication/vortexing step.

10.0 PROCEDURE AND ANALYSIS:

10.1 AOAC Use-Dilution Method with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (MB-05): On a typical test day, one preparation of inoculum is required to inoculate 72 stainless steel carriers necessary to test one product sample. Six of the 72 inoculated carriers are used to estimate the carrier bacterial titer.

10.1.1 Prior to testing, one carrier is randomly extracted from each of 6 Petri dishes (12 carriers/dish).

10.1.2 Place each inoculated carrier into a tube containing 10 mL of letheen broth.

10.1.2.1 Place all tubes with carriers into an appropriately sized beaker and fill the beaker with tap water to the level of letheen broth in the tubes.

10.1.2.2 Hold the beaker in the sonicator so that the water level in the beaker is even with the water level fill line on the sonicator tank and fill the tank up with tap water to the water level fill line. Be sure that the water level in the tank never falls below one inch from the top of the tank.

10.1.2.3 Hold the beaker in the sonicator tank so that it is not touching the bottom and that all three liquid levels (inside the test tubes, inside the beaker and the sonicator tank) are the same.

10.1.2.4 Using an official timer, sonicate carriers inoculated with *P. aeruginosa* and *S. aureus* for 60±5 seconds.

10.1.3 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks (see 16.1).

10.1.4 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5EC until this step can be done. The dilution and plating should be performed preferably within 2 hours after sonication.

10.1.5 For plating, each dilution tube is vortexed briefly prior to removing an

aliquot from the tube. For *S. aureus* and *P. aeruginosa*, 0.1 mL aliquots of the 10^{-1} to10^{-4} dilution tubes are plated. Dilutions are plated in duplicate. The dilution factors on the plates are 10^{-2} to 10^{-5}.

10.1.6 If the spread plate method is used for bacterial enumeration, trypticase soy agar (TSA) plates are prepared in advance and are refrigerated until needed.

10.1.6.1 Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the dry surface of the agar, use a glass, autoclavable or disposable spreading rod and plate spinner until the surface is completely dry.

10.1.7 If the pour plate method is used for bacterial enumeration, the TSA is prepared and tempered after autoclaving (approx. 1 hr) to 45-50EC in a water bath prior to use. Tempered agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.

NOTE: Pour plating is permissible for *S. aureus* and *P. aeruginosa*; however, the dilutions made and plated must be adjusted to account for the larger volume plated, i.e. 1 mL of the 10^{-2} to 10^{-5} dilutions is plated for pour plating.

10.1.8 Incubate plates at 36±1EC for 24-48 hrs.

10.1.9 Colonies may be counted by hand or with the aid of a plate counter. Plates yielding 300 CFU or less are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.2 AOAC Germicidal Spray Products Test and Disinfectant Towelette Test with *S. aureus* and *P. aeruginosa* (MB-06 and MB-09, respectively): On a typical test day, one preparation of inoculum is required to inoculate 72 glass slide carriers. Six of the 72 carriers will be assayed for carrier counts for each inoculum preparation. Slide carriers are randomly selected for carrier counts.

10.2.1 Prior to testing, place each of the inoculated, dried carriers in a 38 × 100 mm culture tube containing 20 mL of medium (e.g., letheen broth). Vortex immediately. Vortexing time is 60±5 seconds for *P. aeruginosa*, and 120±5 seconds for *S. aureus*.

10.2.2 Serial ten-fold dilutions of the vortexed carrier tubes are made in 9 mL dilution blanks (see 16.1).

10.2.3 If the serial dilutions are not made and plated immediately, the tubes are kept at 2-5EC until this step can be performed. The dilution and plating should be performed preferably within 2 hours after vortexing of carriers.

10.2.4 For plating, each dilution tube is vortexed briefly prior to removing an aliquot from the tube. For *S. aureus* and *P. aeruginosa*, 0.1 mL aliquots of the 10^{-1} to 10^{-4} dilution tubes are plated. Dilutions are plated in duplicate. The dilution factors on the plates are 10^{-2} to 10^{-5}.

10.2.5 For spread plate method, see section 10.1.6.

10.2.6 For pour plate method, see section 10.1.7.

10.2.7 Incubate plates at 36±1EC for 24-48 hrs.

10.2.8 Colonies may be counted by hand or with aid of a plate counter. Plates yielding 300 CFU or less are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.3 AOAC Confirmatory Tuberculocidal Test with *M. bovis* (BCG) (MB-07): Three porcelain carriers per inoculum preparation are assayed. For tuberculocidal testing, a total of 24 carriers are inoculated on a typical test day (see SOP MB-07, Confirmatory Tuberculocidal Method). The 24 carriers are distributed in 2 Petri dishes (12 carriers/dish). For the carrier count assay, randomly select three carriers for enumeration.

10.3.1 Place each inoculated carrier in 20 × 150 mm tube containing 10 mL of Modified Proskauer Beck (MPB). The tubes are then placed in a beaker containing tap water up to the level of media in the tubes. Likewise, the level of water in the sonicator bath is adjusted to the same level as the liquid in the tubes and beaker.

10.3.2 Sonicate carriers for 10 minutes. The sonicated tubes with the carriers are referred to as the “sonicated carrier tubes.”

10.3.3 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks.

10.3.4 If the serial dilutions are not made and plated immediately, the sonicated

tubes are kept at 2-5EC until this step can be done (preferably within 2 hours after sonication of carriers).

10.3.5 For plating, each dilution tube is vortexed briefly prior to removing an aliquot from the tube. 0.1 mL aliquots of the 10^{0} to 10^{-3} dilution tubes are plated. Dilutions are plated in duplicate. The dilutions on the plates are 10^{-1} to 10^{-4}.

10.3.6 If the spread plate method is used for bacterial enumeration, Middlebrook 7H9 agar plates are prepared in advance and are refrigerated prior to use. Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, use a glass, autoclavable or disposable spreading rod and plate spinner until the surface is completely dry.

10.3.7 If the pour plate method is used for bacterial enumeration, the Middlebrook 7H9 (M7H9) agar is prepared and tempered (approx. 1 hr) to 45-50EC in a water bath prior to use. Tempered M7H9 agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.

10.3.8 Incubate plates at 36±1EC for a minimum of 21 days (up to 25 days).

10.3.9 Colonies may be counted by hand or with aid of a plate counter. Plates yielding 300 CFU or less per plate are used for enumeration. Plates that have colony counts over 300 will be reported as TNTC.

10.4 AOAC Germicidal Spray Products Test with *M. bovis* (BCG) (MB-07): On a typical test day, one inoculum preparation is used to seed carriers for testing (a minimum of 16 carriers per sample). Three glass slide carriers are evaluated per inoculum preparation per test day.

10.4.1 Prior to testing, place each inoculated, dried carrier in a 38 × 100 mm tube containing 20 mL of Modified Proskauer Beck (MPB) broth. Carriers are selected at random.

10.4.2 Vortex each tube for 15 seconds. These tubes are referred to as Avortexed carrier tubes."

10.4.3 Serial ten-fold dilutions of the vortexed carrier tubes are made in 9 mL dilution blanks.

10.4.4 If the serial dilutions are not made and plated immediately, the sonicated

tubes are kept at 2-5EC until this step can be done (preferably within 2 hours after vortexing of carriers).

10.4.5 For plating, each dilution tube is vortexed briefly prior to removing an aliquot from the tube. 0.1 mL aliquots of the 10^{0} to 10^{-3} dilution tubes are plated. Dilutions are plated in duplicate. The dilutions on the plates are 10^{-1} to 10^{-4}.

10.4.6 For plating methods and incubation conditions, refer to sections 10.3.6 through 10.3.9.

10.5 AOAC Sporicidal Activity Test with *B. subtilis* (MB-15): Refer to MB-15.

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Data will be recorded on data sheets (see 16.2). Calculations will be computed using a Microsoft Excel spreadsheet (see 16.3). Electronic copies of the spreadsheet as well as hard copies will be retained.

11.2 To calculate CFU/mL per carrier:

$$\frac{(avg.\ CFU\ for\ 10^{-w}) + (avg.\ CFU\ for\ 10^{-x}) + (avg.\ CFU\ for\ 10^{-y}) + (avg.\ CFU\ for\ 10^{-z})}{10^{-w} + 10^{-x} + 10^{-y} + 10^{-z}},$$

where 10^{-w}, 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions plated. In the event that one or more dilutions yield plate counts greater than 300, those counts and their corresponding dilutions will not be used in the calculations. In the event that only one of two plates has counts yielding 300 CFU or less, that plate count and its corresponding dilution will be included but no average will be determined.

NOTE: Plate counts of 0 are to be included in all calculations.

11.2.1 To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication or vortexing. Numbers are rounded and only two significant figures are used in calculating averages.

NOTE: Numbers will be rounded upon determination of the CFU/carrier.

11.2.2 Calculate the average CFU/carrier for all carriers tested.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the Carrier Count Data Sheets. Completed forms are archived in notebooks kept in secured file cabinets in the file room D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP=s official retention schedule as stipulated in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate forms (see 16.0).

13.2 If an unacceptable level of contamination occurs, the decision to repeat testing under this scenario is at the discretion of the senior scientist or branch chief.

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 If the average carrier count is less than 1.0×10^4 CFU/carrier for *Staphylococcus aureus, Pseudomonas aeruginosa* and *Mycobacterium bovis* (BCG) and less than 1.0×10^5 CFU/carrier for *Bacillus subtilis*, the associated efficacy test will be deemed invalid and will be repeated.

15.0 REFERENCES:

15.1 SOP MB-05: AOAC Use Dilution Method for Testing Disinfectants

15.2 SOP MB-06: Testing of Spray Disinfectants Against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium bovis* (BCG)

15.3 SOP MB-07: Confirmatory Tuberculocidal Method for Testing Disinfectant Efficacy

15.4 SOP MB-09: Disinfectant Towelette Test Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

15.5 SOP MB-15: Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (*Bacillus* × porcelain component only)

16.0 FORMS AND DATA SHEETS:

16.1 Serial Dilution/Plating Tracking Form

16.2 Carrier Count Data Sheet

16.3 Sample Carrier Count Spreadsheet
MS Excel spreadsheets: Carrier.Count.Template_CTB
Carrier.Count.Template_CTB.Spray
Carrier.Count.Template_UDT
Carrier.Count.Template_UDT.Spray

Serial Dilution/Plating Tracking Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____	
EPA Reg. No.	
Name	

Sample No.	
Test Date	
Organism	
SOP	

Confirmed by: _____	Dilution Tube					
	Tube with carrier[1]	1	2	3	4	5
Volume in dilution tube prior to addition						
Volume added to dilution tube						
Dilution tube						
Volume plated						
Dilution on plate[2]						
Number of plates per dilution						
Media plated onto						
Number of carriers analyzed						
Comments:						

[1]Volume of media in the tube with the carrier will be accounted for in the CFU/carrier calculation.
[2]Dilution on plate based on dilution tube.

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Carrier Count Data Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____	
EPA Reg. No.	

Name	
Sample No.	
Test Date	
Organism	
SOP	

Check the appropriate test type below:

9 AOAC Use Dilution Test
9 AOAC Germicidal Spray Products Test
9 Other – record test type in comments section
9 AOAC Germicidal Towelette Products Test
9 AOAC Confirmatory Tuberculocidal Test

RESULTS				
Date/Initials				
Plating method				
Volume of media in initial tube receiving carrier				
Carrier No. / Dilution	CFU per Dilution Plate (2)			
1	/	/	/	/
2	/	/	/	/
3	/	/	/	/
4	/	/	/	/
5	/	/	/	/
6	/	/	/	/
Comments:				

Sample Carrier Count Spreadsheet
OPP Microbiology Laboratory

Carrier Count Spreadsheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:	
EPA Reg. No.	
Name	
Sample No.(s)	
Test Date	
Organism	
SOP	
Test Type	

Volume of media in tube with carrier (mL): _____

Carrier No. / Dilution	CFU per Plate				CFU/mL per carrier	CFU/carrier*
	1.E-02	1.E-03	1.E-04	1.E-05		
1						
2						
3						
4						
5						
6						
					Average CFU per carrier for all carriers tested:	
Comments:	*CFU/carrier calculation includes volume in the sonicated/vortexed tube with the carrier (10mL or 20mL).					